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Alpha-Amylase Starch Binding Domains: Cooperative Effects of Binding to Starch Granules of Multiple Tandemly Arranged Domains[▽]

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The *Lactobacillus amylovorus* alpha-amylase starch binding domain (SBD) is a functional domain responsible for binding to insoluble starch. Structurally, this domain is dissimilar from other reported SBDs because it is composed of five identical tandem modules of 91 amino acids each. To understand adsorption phenomena specific to this SBD, the importance of their modular arrangement in relationship to binding ability was investigated. Peptides corresponding to one, two, three, four, or five modules were expressed as His-tagged proteins. Protein binding assays showed an increased capacity of adsorption as a function of the number of modules, suggesting that each unit of the SBD may act in an additive or synergic way to optimize binding to raw starch.

Adsorption of amylases is frequently considered a prerequisite for the hydrolysis of insoluble starch (16, 22, 24). For this adsorption, some amylases contain a carbohydrate-binding module (CBM). A CBM is defined as an ancillary module of 40 to 200 amino acids with a discrete fold that possesses carbohydrate-binding activity and is usually contiguous to a carbohydrate-active enzyme. The CBM does not have catalytic activity; it seems to allow the interaction between the insoluble substrate and the solubilized enzyme, bringing the substrate to the active site in the catalytic domain and consequently improving hydrolysis (23). CBMs have been classified into 49 families based on their sequence similarities, according to the CAZy database (http://www.cazy.org; last update on 2 April 2007). Starch binding domains (SBDs) belong to families CBM20, CBM21, CBM25, CBM26, CBM34, CBM41, and CBM45.

Approximately 10% of all amylolytic enzymes possess a distinct domain that facilitates binding to raw starch (13). This independent domain has been identified in such microorganisms as filamentous fungi, gram-positive bacteria, proteobacteria, actinomycetes, and archaea (12). The starch binding function has been demonstrated for only some glycoside hydrolases, α-amylases, cyclodextrin glucanotransferases, and acarviose transferases from glycoside hydrolase family 13 (GH13), β-amylases from GH14, and glucoamylases from GH15, while in most it remains unverified (18).

The presence of SBDs has been demonstrated in three α-amylases from lactobacilli (10, 20, 22). The genes encoding these α-amylases (*amyA*) have been sequenced (11, 20). Amino acid sequence analysis of these enzymes reveals an identity of more than 96% and a structure that comprises at least two discrete functional domains, the N-terminal or catalytic domain (GH13) and the C-terminal domain or SBD (CBM-26) formed by direct tandem repeat units; four modules have been found in *Lactobacillus plantarum* and *Lactobacillus manihotivorans* α-amylases and five modules in the *Lactobacillus amylovorus* enzyme. In a previous report, we have shown that the protein encoded by the *L. amylovorus amyA* gene, which lacks tandem repeats, is unable to adsorb and hydrolyze raw starch despite the fact that it can hydrolyze soluble starch almost as well as complete α-amylase (22). After establishing the role of tandem repeats as SBDs, we identified in each module some of the residues reported as part of the carbohydrate binding sites in others SBDs; therefore, one single module is supposed to be able to bind raw starch; this capability was confirmed experimentally (26).

The observed modular arrangement, notably the presence of four or five CBM26 tandem units, represents an unusual architecture of the SBD related to amylases from family GH13. Nevertheless, two SBD modules (one from CBM25 and the other from CBM26) have recently been described for the maltohexaose-forming amylase from *Bacillus halodurans* (5). Two SBD modules, both from CBM25, were also found in the α-amylases from *Bacillus* sp. no. 195 (27) and *Clostridium acetobutylicum* ATCC 824 (21).

In this paper, we present an analysis of the functionality of the C-terminal direct tandem repeats of *L. amylovorus* α-amylase, using His-tagged derivatives of the entire SBD or one, two, three, or four modules. Their binding capacity and its

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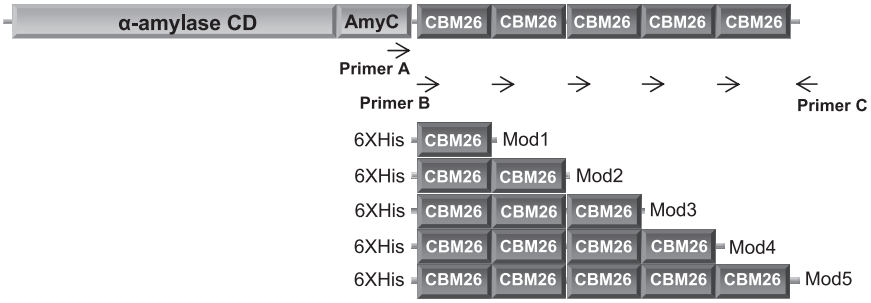

FIG. 1. PCR primers and molecular architecture of *L. amylovorus* α-amylase. Priming of oligonucleotides designed for PCR amplification of DNA encoding tandem CBM26 modules. Sequences of the primers are included in the text. α-Amylase CD, α-amylase catalytic domain; AmyC, α-amylase domain C.

possible cooperation were investigated with the aim of proposing a possible biological role.

MATERIALS AND METHODS

Microbial strains and media. *Escherichia coli* strain DH5α [*supE44* Δ*lacU169* (ϕ80*lac*ZΔM15) *hsdR17 recA1 endA1 gyr96 thi-1 relA1*] was used as the cloning host, and *E. coli* strain M15 (QIAGEN) (cells contain the pREP4 plasmid encoding the lac repressor in *trans*) was used for protein production. All strains were grown in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) supplemented with ampicillin (100 mg/liter) and kanamycin (25 mg/liter).

Lactobacillus amylovorus NRRL B-4540 was kindly provided by the ARS Culture Collection, U.S. Department of Agriculture, Peoria, IL. The strain was grown in MRS medium-starch (2%) at 30°C (9).

Construction of plasmids. All DNA manipulations were performed using standard protocols (25). To produce different peptides equivalent to one, two, three, four, or five repeats, five different fragments were amplified from the region contained between amino acids 444 and 953 of the *L. amylovorus amyA* gene (GenBank accession number U62096) with the following primers: primer A, 5′-TGAAAACAAGGCTGGTTCAG-3′; primer B, 5′-GATGAACTGCTG GTATCGGC-3′; and primer C, 5′-TACTACCCCAACTTGAAGGC-3′. The conditions used in the PCRs were previously described (11). The amplified fragments were purified and ligated to pGEM-T Easy vector (Promega), following the manufacturer's instructions, and transformed into *E. coli* DH5α by electroporation (25).

Gene expression and peptide purification. Obtained pGEM-T-easy constructs were digested with EcoRI, and released fragments were treated with mung bean nuclease and cut with BamHI. Gene derivatives were introduced into pQE31 (QIAGEN) treated with SmaI and BamHI. pQE31 carries six codons encoding His_6. Five different gene fusions were constructed and verified by sequencing (Laragen, Inc). The first encoded the protein 1-Mod, containing one of the repeated units and the His_6 tag at the N terminus. The second encoded 2-Mod, containing two units and the His_6 tag at the N terminus. 3-Mod, 4-Mod, and 5-Mod contained three, four and five units, respectively. Constructs were electrotransformed into *E. coli* M15 for protein expression.

The strains were grown in LB medium supplemented with the required antibiotics for 10 h at 37°C. Expression of proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM, and incubation was continued at 30°C for 8 h. Cells were harvested (8,000 × *g* for 10 min at 4°C) and washed in 20 mM Na_2HPO_4 at pH 7.4 before lysis. The obtained proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15) and identified by Western blotting with a primary anti-His_6 antibody (Roche) and a secondary anti-mouse immunoglobulin G antibody labeled with alkaline phosphatase (Perkin-Elmer Life Sciences), according to the manufacturer's instructions.

For protein purification, the *E. coli* pellet was resuspended to about 1/100 of the original culture volume in 20 mM Na_2HPO_4, 500 mM NaCl, and 50 mM imidazole at pH 7.4, with protease cocktail inhibitor (SIGMA). Cells were disrupted by sonication three times for 20 s at 60 Hz (Vibra-Cell; Sonics & Materials, Inc.). Obtained polypeptides were purified from clarified extract by immobilized metal affinity chromatography according to the manufacturer's protocols (Pharmacia Biotech).

***Lactobacillus amylovorus* α-amylase purification.** Following an 18-h batch culture (250 ml), the fermentation broth was collected and centrifuged at 9,000 × *g* for 15 min at 4°C. The *L. amylovorus* NRRL B-4540 α-amylase was purified from the supernatant by affinity chromatography as previously described (22), using a β-cyclodextrin–epoxy-activated Sepharose 6B column (16 by 35 mm). After being washed with 0.1 M citrate-phosphate buffer, pH 5.5, the bound amylase was eluted with 8 mM β-cyclodextrin in the same buffer at a flow rate of 0.5 ml min^{-1} for 400 min.

Electrophoresis analyses. SDS-PAGE (7.5 to 12%) was performed according to the method of Laemmli (15). Proteins were visualized by Coomassie blue staining as described by Blakesley and Boezi (2).

Binding assay. Before the binding assays, proteins were dialyzed extensively against 0.1 M citrate-phosphate buffer, pH 5, and purity was assessed by SDS-PAGE (15). Various amounts of peptides were added to a prewashed raw-cornstarch suspension (final concentration, 1%) in 0.1 M citrate-phosphate buffer, pH 5, to a final volume of 60 μl. The mixture was incubated at 4°C for 30 min with gentle shaking (20 rpm) and centrifuged at 13,000 × *g* for 5 min. The protein concentration in the supernatant was subtracted from the total protein to calculate the total amount of protein bound (1).

The protein concentration was determined by measuring the absorbance at 280 nm with the theoretical molar extinction coefficient of 20,416, 46,870, 62,800, 80,080 or 99,700 liter mol^{-1} cm^{-1} for 1-Mod, 2-Mod, 3-Mod, 4-Mod, or 5-Mod, respectively, or 207,680 for the complete α-amylase (8).

RESULTS AND DISCUSSION

Construction and purification of SBD modules. The *L. amylovorus* α-amylase gene encodes a 953-amino-acid protein with a predicted molecular mass of 105 kDa. The mature form of the protein consists of a GH13 N-terminal catalytic domain and a C-terminal SBD composed of five identical modules (CBM26) in tandem. In order to study the role of these repeats, various deletion derivatives were constructed, as summarized in Fig. 1.

Plasmids carrying the five tandem modules (the entire SBD) and the separated modules were introduced into *E. coli* M15 for production. After culture, the proteins were released by cell sonication and purified by metal affinity chromatography.

Adsorption to granular starch. Fig. 2 shows the obtained adsorption isotherms. The amount of protein bound depends not only on the initial protein concentration but also on the number of modules constituting the peptide. One module is capable of adsorbing to starch granules. This ability increases as the number of modules increases, until reaching the five modules forming the *L. amylovorus* α-amylase SBD, which strongly adsorbs cornstarch, even more than the whole amylase. The inability of the α-amylase catalytic domain to bind to and hydrolyze raw starch in the absence of the SBD has previously been demonstrated (22).

The affinity of these modules for starch was analyzed by

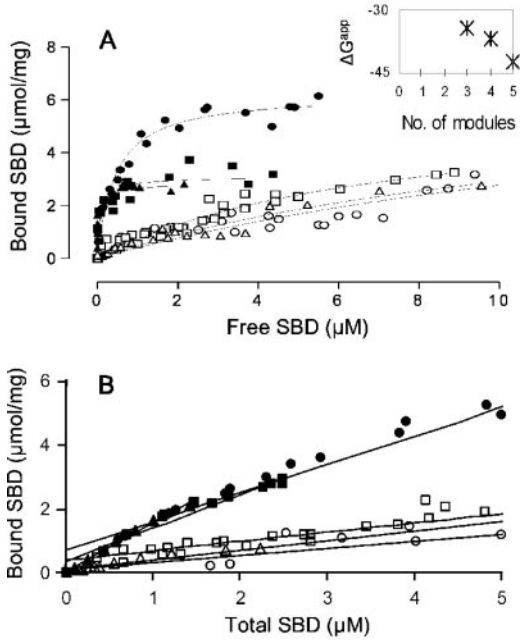

FIG. 2. Adsorption isotherms (tandem units) for CBM26 SBD. (A) Binding curve, considering free protein after equilibrium; (B) initial adsorption isotherms; slopes describe partitioning of proteins between solid and liquid phases at low surface coverage. ○, 1-Mod; □, 2-Mod; ●, 3-Mod; ■, 4-Mod; ▲, intact SBD; △, *L. amylovorus* α-amylase. Adsorption to cornstarch at 4°C in 0.1 M citrate-phosphate buffer, pH 5, was determined as described in Materials and Methods.

binding isotherm measurements in accordance with the following Michaelis/Langmuir relationship:

$$q_{ad}/q = Kp \times q_{max}/(1 + Kp \times q)$$

where q_{ad} is the extent of protein adsorbed (μmol of protein/mg of starch), q is the free protein in solution (μM), Kp is a constant, and q_{max} is the maximum amount of protein adsorbed by the substrate (14).

The Langmuir equation presumes that the adsorption surface is homogeneous and that no interactions exist between adsorbing molecules. Although structural heterogeneity and interactions between adsorbed molecules are probably important factors in starch adsorption, the Langmuir relationship is the most commonly employed equation for describing the phenomena of adsorption of glycoside hydrolases to their substrates (1, 3, 14, 19, 27). The binding parameters for three, four, and five modules and of whole amylase are summarized in Table 1. The association constant increased with the number of modules present in the peptide, but in the case of the whole amylase, simultaneous interaction of the five CBMs seemed to be unfeasible.

The maximum amount of protein adsorbed, q_{max}, seems to be linked to the size of the protein (Fig. 3), given that the bound protein was directly proportional to the available surface on the starch granule, a condition previously reported for cellulose (7). The decrease in the q_{max} value with an increase in molecular weight can be described by the equation included in Fig. 3. In addition, when the Langmuir model was used for the 1-Mod and 2-Mod adsorption data,

TABLE 1. Affinities of CBM26 tandem modules for cornstarch as determined by depletion isotherms

Protein	*Kp* (μM)	q_{max} (μmol protein/mg starch)	*K* value	K_{SBD}/K_{mod}	SB
1-Mod			0.21 ± 0.011	7.08	
2-Mod			0.29 ± 0.011	5.28	0.69
3-Mod	3.38 ± 0.001	6.02 ± 0.002	0.89 ± 0.047	1.72	1.41
4-Mod	8.20 ± 0.005	3.27 ± 0.002	1.06 ± 0.07	1.44	1.68
SBD	90.9 ± 0.015	2.71 ± 0.0005	1.53 ± 0.06	1	2.43
α-Amylase	1.28 ± 0.004	1.20 ± 0.004	0.41 ± 0.02	3.73	

fitted curves did not correlate with experimental results and correlation coefficients were rather low. In both cases, saturation was not achieved even at a peptide concentration of 20 μM. The relationship appears to be linear, probably due to interactions between the proteins. For this reason, it is not possible to calculate the contribution of each module to adsorption, and the apparent free-energy change could only be estimated from the equation $\Delta G^{app} = -RT \ln Kp$ (where ΔG^{app} is the apparent ΔG, R is the ideal gas constant, and T is temperature) for three, four, and five modules and whole amylase (Fig. 2, inset). The ΔG^{app} values calculated show a decrease in free energy (increasing affinity) with an increasing number of modules, with the exception of the whole amylase, the ΔG^{app} value of which indicated a minor affinity compared to that observed in the peptide with three modules.

Therefore, to compare the adsorption between all peptides, we decided to analyze the initial slopes of the isotherms shown in Fig. 2B, where lateral interactions between the adsorbed peptides do not influence binding. The data were fitted to the following linear equation:

$$[E_b] = K[E_t]$$

where $[E_b]$ is the concentration of SBD modules bound to the substrate (μmol/mg substrate), K is the linear coefficient (dimensionless), and $[E_t]$ is the total concentration of SBD modules in the reaction system (μM).

The linear coefficients are presented in Table 1. Binding levels of individual CBM peptides were compared by calculating the ratio of K_{SBD} to the K value of every peptide constructed (Table 1). These ratios demonstrated that each pep-

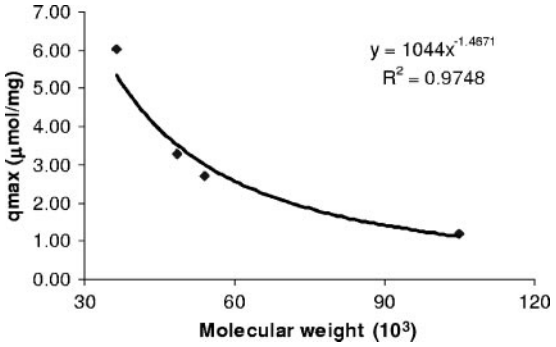

FIG. 3. q_{max} values of CBM26 tandem modules as a function of their molecular weights when binding to cornstarch.

tide did not contribute equally to starch granule adsorption and confirmed that whole amylase did not bind to the substrate with all five modules present in its structure. In other words, a degree of synergy based on the initial binding of components (*K*) may be established as follows:

$$SB = [K_{X\,\mathrm{mod}}] / \sum_{5}^{i=1} [K_{\mathrm{single\ mod}}]_i$$

where SB is synergistic binding, $K_{\mathrm{X\ mod}}$ is the *K* value for two, three, four, or five modules, and $K_{\mathrm{single\ mod}}$ is the *K* value for one module; an SB value greater than 1 indicates an enhancement in the adsorption of the subsequent module when a previous one was bound. Table 1 summarizes the SB value for each peptide; the values are greater than 1 for three, four, and five modules, showing that when combined in the same polypeptide chain, the modules display a significant increase in affinity for insoluble substrate.

Affinity improvement has also been reported in the SBD of the maltohexaose-forming amylase from *Bacillus halodurans*. The enhanced affinity is attributed to the simultaneous interaction of the two tandem CBMs present in the enzyme (one from family CBM25 and the other from family CBM26) through an avidity effect (5), a term originally used to describe the strength of binding of a molecule with multiple binding sites by a larger one, particularly the binding of a complex antigen by an antibody. Similar performances were also reported for some cellulases and xylanases (4, 6, 17). Nevertheless, this is the first report of a five-tandem-module SBD analyzed not only as separated modules but also as an element of the whole amylase.

Concerning the catalytic domain, a steric hindrance may explain why whole amylase adsorbs to starch with only two or three modules. Occasionally, amylases have the catalytic domain linked to the binding domain by a linker region of considerable internal mobility (28), but the *L. amylovorus* α-amylase does not present any linker structure between the catalytic domain and the SBD, nor among the modules. As a result, some of the modules may act as linkers. In conclusion, we propose that any of the CBMs may bind to starch with equal affinity by both intrachain and interchain interactions. Consequently, subsequent binding events are facilitated by previously established interactions. As a result, the number and flexibility of the neighbor CBMs in the molecule should determine the affinity of the SBD.

The origin of multiple binding modules remains unclear. It has been suggested that duplication of CBMs could be an evolutionary mechanism of thermophilic microorganisms to compensate for a loss of binding affinity in their glycoside hydrolases at elevated temperatures (6). An analysis was carried out with all the starch binding modules listed in the CAZy database (http://www.cazy.org), corresponding to families CBM20, -21, -25, -26, -34, -41, and -45. From the 504 entries, 2 or more modules were found only in 40 sequences, most of them from streptococci and lactobacilli. Given that lactic acid bacteria are mesophile microorganisms, module multiplication cannot be justified by growth temperature. The presence of tandem modules is likely an evolutionary adaptation designed to allow degradation of raw starch by improving lactobacillus α-amylase binding to the starch granules contained by vegetal material.

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